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Studying kinetochore kinases

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Abstract

Mitotic kinetochores are signaling network hubs that regulate chromosome movements, attachment error-correction, and the spindle assembly checkpoint. Key switches in these networks are kinases and phosphatases that enable rapid responses to changing conditions. Describing the mechanisms and dynamics of their localized activation and deactivation is therefore instrumental for understanding the spatio-temporal control of chromosome segregation.

Key words: kinetochore, kinase, small molecule inhibitor, antibody, signaling, phosphorylation, spindle checkpoint

1. Introduction

Named by Lester Sharp in the early 1930's for their apparent ability to power chromosome movements, kinetochores are the business ends of chromosomes during mitosis. They are large protein assemblies that connect chromatin to the mitotic spindle which is tasked with dragging identical, duplicated chromatids in opposite directions. Kinetochores are therefore the focal points of signaling networks that regulate chromosome-spindle attachments and cell cycle progression (1-3) .

Prometaphase is the time of mitosis during which the chromosomes attempt to become bioriented, that is, to have each sister from a pair of duplicate chromosomes attach to microtubules from opposite spindle poles. In mammalian cells, this process is quite error-prone. In fact, many initial attachments are of the so-called syntelic kind and are in need of correcting (4-6). Consequently, error-correction mechanisms have evolved that enable iterative rounds of detachment-attachment until a chromosome is bioriented. All the while, anaphase initiation cannot be permitted. Surveillance of attachment/biorientation and transmission of that information to the cell cycle machinery is the primary job of the spindle assembly checkpoint (SAC). The combined actions of the error-correction and SAC machineries therefore prevent erroneous chromosome segregations (7, 8).

As with most signaling networks wired to achieve speed and responsiveness, kinases and phosphatases are at the heart of attachment error-correction and the SAC. The kinases Aurora B, MPS1, BUB1, PLK1, and CDK1, and the phosphatases PP1 and PP2A-B56 all have major contributions. Several excellent reviews have summarized our current understanding of their molecular workings, and we therefore refer the reader to those for detailed insights (1-8). For the purpose of this chapter, however, the following is relevant; localization of BUB1 to human kinetochores is crucial for SAC activity as it ensures localized production of the wait-anaphase signal. BUB1 recruitment is induced by MPS1, which phosphorylates multiple repeated motifs in the kinetochore scaffold KNL1 to create phospho-docking sites for the BUB1 adaptor BUB3 (figure 1). The axis Aurora B - MPS1 -

BUB1 is not only at the heart of the SAC, it is also the core of attachment error-correction: Aurora B phosphorylates microtubule-binding proteins such as NDC80/HEC1 to allow destabilization of erroneous attachments (figure 1). MPS1 ensures localized Aurora B activity and likely has other targets required for efficient error-correction. Finally, BUB1 promotes Aurora B localization by phosphorylating histone H2A on T120. Therefore the error-correction and SAC pathways are extensively entwined, and this is further exemplified by the fact that the phosphatases PP1 and PP2A-B56 counteract both error-correction and the SAC to stabilize chromosome spindle attachments and allow anaphase onset (9-14).

Recent years have seen much advance in our mechanistic understanding of localized activation and inactivation of kinetochore kinase signaling pathways. This is largely due to reagents that allow immediate inhibition of kinases, and reagents to monitor kinase output at high spatial and temporal resolution. This chapter describes how reagents such as small molecule inhibitors and phospho-specific antibodies can be used to draw meaningful conclusions about kinetochore kinase function.

2. Material

2.1 Visualising and quantifying kinase localisation and activity – fixed analysis

1. High precision 1.5H 12 mm coverslips.
2. Poly-L-lysine solution (Sigma Aldrich). Dilute 1:10 in PBS (0.01% w/v) just prior to coating wells.
3. Nocodazole (Millipore or Sigma Aldrich). Make 1 mg/ml stock in DMSO and store at -20°C.
4. Paclitaxel (Taxol; LC Labs). Make 1 mM stock in DMSO and store at -20°C.
5. Noscapine (Tocris Biosciences). Make 50 mM stock in ddH₂O and store at -20°C.
6. S-Trityl-L-cysteine (STLC; Sigma Aldrich). Make 10 mM stock in DMSO and store at -20°C.
7. Monastrol (Tocris Biosciences). Make 200 mM stock in DMSO and store at -20°C.

8. MG-132 (Sigma Aldrich). Make 5 mM stock in DMSO and store at -20°C.
9. Pro-TAME (Boston Biochem). Provided as a 20 mM stock in DMSO.
10. Thymidine (Sigma Aldrich). Make 200 mM in H₂O, store at room temperature and use within 2 weeks (see note 1).
11. Pre-extraction media (PEM). 100 mM PIPES (pH 6.8), 1 mM MgCl₂, 5 mM EGTA, 0.2% Triton X-100 (all Sigma Aldrich). Mix fresh immediately prior to use.
12. Paraformaldehyde (Sigma Aldrich). Use the prilled version to prevent noxious powder from being released when weighing (see note 2).
13. 245 mm square plates (Corning). For use as an antibody incubation chamber.
14. Parafilm. 100 mm wide for antibody incubations.
15. Various primary antibodies, listed in Table 1.
16. Alexa Fluor® range of fluorescent secondary antibodies (Life Technologies). Use highly cross-adsorbed versions wherever possible to limit cross-reaction.
17. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Aldrich). Make 1 mg/ml stock in H₂O and store at -20°C. Store working stock at 4°C for up to 3 months.
18. Microscope slides (72 x 25 mm).
19. ProLong® Gold antifade mounting media (Life Technologies).
20. Deltavision Elite deconvolution microscope (Applied Precision) equipped with a 100 X 1.4 numerical aperture (NA) objective and a Coolsnap HQ2 (Photometrics) CCD camera. Other similar automated microscope setups capable of multi-position imaging and high precision Z-sectioning could perform equally well.
21. Fiji ImageJ. Open source image analysis software (download from <http://fiji.sc/Fiji>).

2.2 Visualising and quantifying kinase localisation – live imaging

1. 8-well chamber -slide (ibidi)

2. Syringe and 23G needle
3. Leibovitz-15 (L-15) medium supplemented with 10% FBS and 1% PenStrep (all Life Technologies).

2.3 Modulating kinetochore kinase activity

A number of different kinase inhibitors have been developed to target kinetochore kinases. In addition, various gatekeeper kinase mutants have also been developed using the Shokat approach to achieve specific kinase inhibition (See Jallepalli et al in this volume) (18). These are listed in Table 2.

3. Methods – fixed cells analysis

3.1 Determining the correct experimental conditions

Kinetochore kinase activity is always affected (directly or indirectly) by kinetochore-microtubule attachment or tension. Therefore, as mitosis progresses the kinase activity at individual kinetochores will change as kinetochore-microtubule attachments form. For this reason, it is usually advisable to reduce this variability by performing experiments in the presence of nocodazole to inhibit microtubule polymerisation (see note 3).

To measure the effects of microtubule attachment on kinetochore kinase activity directly, various inhibitors can be used to enrich cells at different stages of kinetochore-microtubule attachment (as outlined in table 3)

If required, the total number of mitotic cells can be further enriched by thymidine synchronisation prior to inhibitor treatment (see note 5).

1. Arrest cells for 24 h in full growth media and thymidine (2 mM).
2. Wash cells 1X in PBS and release into full growth media for 6 h
3. Add fresh media containing required drugs (except for MG-132 which prevents mitotic entry and so must be added 8-12 h after the thymidine release)
4. Cells will be enriched in mitosis after a total of 8-12 h following thymidine release (depending on cell type).

3.2 Setting up a fixed-cell imaging experiment.

1. Transfer coverslips to 24 well plates using a vacuum aspirator to transfer sterile coverslips inside a tissue culture hood.
2. Plate cells onto coverslips at required density in 0.5 ml media and then immediately press coverslip down with a pipette tip to ensure no air is trapped beneath (see note 6).
3. Allow at least 24 h for cells to attach, but often longer depending on experimental conditions (siRNA knockdown, synchronisation etc; see note 7).
4. When the particular mitotic state is achieved, cells should be fixed, with or without prior pre-extraction with PEM to decrease the cytosolic signal (see note 8).
5. If pre-extraction is required, remove media and add 0.5 ml PEM for 60 s before adding 0.5 ml 4% PFA for an additional 2 min (see note 9).
6. Remove media or PEM/PFA and add 4% PFA for 10 min (see note 10)
7. Wash 3X in PBS.

3.3 Staining with total and phospho-specific antibodies.

1. Block coverslips in PBS + 3% BSA + 0.2% triton (0.5 ml/well) for at least 30 min.

- 2 Replace blocking solution with PBS.
- 3 Dilute primary antibodies in PBS/3% BSA (see note 11)
- 4 Add 40 µl of primary antibody mix per coverslip onto parafilm (see note 12). Place coverslip on top (cells facing down) and leave at least 2 h at room temperature, but generally overnight at 4°C.
- 5 Transfer coverslips back into the 24 well plate (cells facing upwards) and wash 3X in PBS for 10 min each on a shaking platform.
- 6 Dilute secondary antibodies (1:1000 of the Alexa Fluor® from Life Technologies) and DAPI (1:1000 of 1 mg/ml stock) in PBS + 3% BSA. Place coverslip on 40 µl, exactly as for the primary incubation, but just leave at least 2 h at room temperature in the dark (see note 13).

The kinetochore markers are usually stained with Alexa Fluor® 647, which allows the test proteins to be stained with the visible wavelengths.
- 7 Transfer back into 24 well plate (cells facing upwards) and wash 3X in PBS for 10 min each.
- 8 Dip coverslips in absolute ethanol and place cells face up on tissue paper to dry (for about 5 min).
- 9 Mount coverslips on slide in a small drop of ProLong® Gold antifade (Life Technologies) and leave overnight to set.

3.3 Imaging

1. The experiments were performed on a Deltavision Elite deconvolution microscope described in materials (Applied Precision). Other microscopes equipped with a motorised XY-stage and an accurate Z-stage motor may perform equally well.
2. For each particular stain, allow enough time to image the full set of treatment groups with the same settings on the same day. This will ensure intensity variations are not artificially induced during sampling.

3. Start with the coverslip that is predicted to have the maximal signal intensity (this is often the control sample).
4. Store the XY positions of 10-20 mitotic cells by visualising the DAPI signal through the eyepiece (see note 14).
5. Switch the light path to the camera and change exposure settings for each channel to give a maximal kinetochore signal intensity of 1000-1500 (see note 15).
6. Switch to the far-red channel to visualise the Alexa Fluor® 647 kinetochore signal (either ACA or Cenp-C). Move to position 1 and use the software to adjust the Z position to just below the lowest kinetochore and update the position.
7. Repeat for each stored XY position without changing the manual focus on the microscope body.
8. To simplify quantification choose the channels to be scanned in the same order every time. Our macros (see 3.4) are predesigned for the order: DAPI, test antibodies (488 and/or 568), kinetochore marker (647).
9. Take 43 x 0.2 μ M sections upwards (from the stored z-position) for each XY position. Choose the option in Softworx to automatically deconvolve images and make maximum intensity projections of the deconvolved stacks.

3.4 Quantifying kinetochore intensities

Kinetochore intensities can be quantified in fiji imageJ by using a macro to automate the steps highlighted below. The aim is to quantify the kinetochore intensities with the test antibody as a ratio of the kinetochore marker intensity (after correcting each channel for background signal). Contact either the Saurin or Kops labs for macros predesigned for this purpose (as used in (16)).

1. Open the deconvolved maximum intensity projection using fiji imageJ (see note 16).

2. Use crop to remove any non-mitotic cells in view.
3. Create a selection of the DAPI signal using the “auto-threshold” command followed by “create selection”. Use “add to manager” to save this selection.
4. Use “duplicate” to open the image again in a new image window.
5. Move to the last channel in the new window, use the “convolve” filter to clearly define the kinetochores and create a selection of these kinetochores using the auto-threshold command, as previously. Enlarge the selection by 2 pixels (see note 17) and “add to manager”. This is the kinetochore region [KT].
6. Use the “make inverse” command to choose a selection outside of all kinetochores and “combine” with the DAPI selection to define a region outside of the kinetochores but inside the DAPI. This chromatin area can be used as the background region [BG] for most kinetochore kinases and substrates (see note 18).
7. Close the duplicated window.
8. In the original image window, measure the mean [KT] and [BG] intensity for all channels.
9. Calculate KT intensities for the test antibody using the formula: $[KT-BG]_{\text{test antibody}} / [KT-BG]_{\text{kinetochore marker}}$ (see note 19).
10. The change in KT intensity upon treatment can be calculated by: $\text{Mean KT intensity (control)} / \text{Mean KT intensity (treatment group)}$ (see note 20).
11. The mean and standard deviation of this ratio can then be calculated between individual experiments for statistical purposes.

4. Methods – live cell imaging

4.1 Setting up a live-cell imaging experiment.

1. Plate cells into 8-well chamber slides (Ibidi) using 250 μ l of media per well. Plate the same number of cells as for a standard well of a 24 well plate.
2. On the day of the experiment, exchange media to L-15 (Leibovitz) media with serum for at least 3 h prior to imaging (mitotic entry is inhibited for the first few hours after switching to L-15; see note 21).
3. Turn on the microscope heating unit to equilibrate the microscope to 37°C for at least 2 h prior to filming (see note 22).
4. Secure the chamber slide into position in the stage holder and allow to equilibrate to temperature (see note 23).
5. Determine the required exposure time and, if possible, try to minimise toxicity by sacrificing resolution using binning (see note 24).
6. Run a test on a sample field of view to determine whether the chosen settings are suitable and that the level of bleaching is acceptable (see note 25).
7. Start experiment and check again after a few time points to ensure there has been no focal drift (see note 26).

4.2 Adding small molecules during time-lapse imaging.

Adding and washing out drugs is possible during a time-lapse imaging experiment, but care must be taken not to move the chamber slide or dislodge the loosely attached mitotic cells. Also, if drugs are to be added to different wells from the start of a timelapse movie using the ibidi chamber slides, then this should be performed when the slide is positioned securely on the microscope and not in the tissue culture hoods (see note 27).

1. Make sure the chamber slide is securely placed in position by ensuring the spring loaded slide holder is under sufficient tension (see note 28).

2. Remove the tight fitting lid from the chamber slide and place upside down to allow easy removal with minimum disturbance during filming.
3. Start movie for the desired number of timepoints prior to drug addition and then pause.
4. To remove media use a 5 ml syringe coupled to a 23G needle to withdraw media from the far corner of the well (as far away from the filming position as possible; see note 29).
5. Add 250 μ l media containing drugs gently down the same corner of the well using a 1 ml pipette.
6. Replace lid and continue filming (see note 30).

5. Notes

- 1 Check before use and if crystals have formed then make fresh. Store in a warm place to prevent crystallization.
- 2 Dissolve at 4% in PBS by incubating for approximately 2 h at 60°C with occasional stirring. Do not to heat above 60°C since paraformaldehyde will degrade at high temperatures. Store at 4°C and use within 2 days. Always warm to room temperature prior to use.
- 3 This is particularly important if the chosen treatments are known to affect kinetochore/microtubule attachments, since these would otherwise indirectly affect kinase activity.
- 4 Keep time of drug addition short because extended metaphase durations can lead to premature sister chromatid splitting due to cohesion fatigue (38, 39)
- 5 This type of enrichment will induce variability in the length of time individual cells have spent in mitosis, which can indirectly impact on phosphorylation levels of some substrates (12). To reduce variability, treat with inhibitors for a fixed duration in non-synchronised cells.

- 6 If cells adhere poorly to glass then coat coverslips in poly-L-lysine solution for at least 1 h prior to addition of cells (remove and wash 3x in PBS just before cells are added).
- 7 If experimental conditions are likely to alter the duration of mitosis then this could also indirectly affect substrate phosphorylation (see note 5). If required, treat with the Cdk1 inhibitor RO-3306 (10 μ M) for 2 h (to induce mitotic exit and prevent new mitotic entry). Wash cells 3X in PBS and replace with fresh media (-/+ inhibitors, if required) for a fixed duration to standardise the length of time in mitosis (allow at least 15 min for mitotic entry following RO-3306 washout).
- 8 For kinases or substrates that give a cytoplasmic signal, visualisation of the kinetochore signal can be improved by pre-extraction for 1 min in a triton-containing buffer (PEM) to permeabilise the membrane and remove cytosolic proteins.
- 9 Take care not to dislodge mitotic cells, which are very loosely attached in the triton-containing PEM buffer. The addition of PFA before the PEM is removed is important to prevent inadvertent loss of cells.
- 10 For most antigens we find standard PFA fixation is sufficient, but a small number may benefit from alternative fixation methods. If the signal intensity is low, then try fixation in either methanol or methanol-acetone (50/50) at -20°C for 5 min. This fixes cells by protein denaturation which may reveal antibody epitopes that are otherwise obscured.
- 11 A typical experiment will contain a kinetochore marker (human-ACA or Guinea Pig Cenp-C; both give strong signals and allow co-staining with mouse and/or rabbit antibodies) and a total kinase and/or phospho-substrate antibody (see table.1). For phospho-specific antibodies incubate with dephospho-peptide whenever possible, since we find this increases the phospho-specific signal even for antibodies that have been purified by phospho-peptide enrichment.
- 12 Use a corning 245 mm square tissue culture plate for antibody incubations. Place a strip of parafilm (100 mm thickness) across the plate and draw a grid to represent a 24 well plate.

Place antibody drops directly onto the parafilm and perform coverslip incubations with the lid on to prevent evaporation.

- 13 We use the highly cross-adsorbed options (goat anti-guinea pig for Cenp-C or goat anti-human for ACA) because these show no cross-reaction with mouse/rabbit. If using sheep primary antibodies then use secondary antibodies raised in donkey, because we see significant cross-reaction with secondaries raised in goat.
- 14 Choose cells that are separated from the surrounding cells to simplify the quantification.
- 15 This allows for a 3-fold increase in signal before the 12-bit Coolsnap-HQ2 camera is overexposed. If this is set on the treatment group with the highest predicted kinetochore intensity this is usually sufficient to account for inter-cell variability and prevent overexposure (unless variability is particularly high, for example following transient protein overexpression).
- 16 We routinely use maximum intensity projections, but it is also possible to use sum intensity projections to include all data from every Z-projection. This may be especially important if kinetochore volume changes occur between samples/treatments. Note that cytoplasmic background could then begin to obscure KT signals, if this is the case, then this can be reduced by removing sections above/below the kinetochores prior to projection.
- 17 This selection should now encompass the kinetochore signal in the test antibody channel, but if the test sample is larger (as with Aurora B for example, which gives a diffuse signal around the kinetochore) then increase this number, as required.
- 18 If the kinase or substrate localises to chromatin (e.g Aurora B, pH2A etc.) then choose a ring around the DAPI selection (i.e. cytoplasm) as the background region instead.
- 19 Expressing the intensities as a ratio of an internal kinetochore control allows normalisation against changes in kinetochore size and/or antibody signal intensity, both of which can vary between cells and between experiments.

- 20 Expressing data as a fold-change following treatment normalises against variation in the relative signal intensity of target vs kinetochore marker, which can otherwise vary considerably between experiments.
- 21 L-15 (Leibovitz) is a CO₂-independent media, but standard media (minus phenol red to prevent autofluorescence) can be used if a 95% air/5% CO₂ line is available at the microscope.
- 22 If a large number of live microscopy experiments are performed then keep the microscope unit at 37°C permanently. This will prevent downtime without affecting the quality of the fixed analysis.
- 23 Temperature fluctuations will cause the focal point to drift, therefore allow enough time for the temperature to stabilise and check the focus is steady for at least 5-10 mins before filming.
- 24 By using 2x2 binning the resolution is decreased 4-fold with a corresponding 4-fold increase in fluorescence signal. 4x4 binding leads to 16-fold changes, which can be very important to increase low signal intensities if detailed resolution is not required.
- 25 For example, if running a time series of 100 time points with 3 x Z sections every 5 min, then quickly image one area of cells with those settings (but without the time series delay). Use the data inspector in Softworx to measure fluorescence decay throughout the course of the 100 images and try to ensure this is no more than 10%.
- 26 If focal drift has occurred, use the microscope focus dial to update the correct focus in one position. This should correct all the other positions since they are all relative.
- 27 The ibidi chamber slides have a very low depth to the wells and we have frequently observed mixing between the wells during transport (i.e. from tissue culture room to the microscope). This can be minimised by using very low volumes of media (150 µl per 8 well chamber), however we have found the safest option is to only add drugs when the chamber slide is securely positioned on the microscope stage.

- 28 If using different stage inserts then we find that blu-tack (or a similar flexible adhesive) is excellent for preventing movement during filming.
- 29 Cut off the end of the needle to remove the bevelled edge and bend slightly. This will allow full removal of media when placing the needle into the corner of the well so that it just touches the base of the well.
- 30 If washout is required, replace lid upside down and repeat same procedure at the time of drug washout.

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Figure legends

Figure 1. Network model of kinase/phosphatase signaling at kinetochores. A selected set of kinases and phosphatases that control error-correction and the SAC are indicated along with some of their relevant substrates. Solid lines indicate direct connections, dashed lines indicate indirect ones.

Table 1. A list of kinetochore antibodies that have been verified to work by immunofluorescence.

Table 2. A list of inhibitors against different kinetochore kinases

Table 3. A list of Inhibitors that can be used to modulate kinetochore-microtubule attachment status

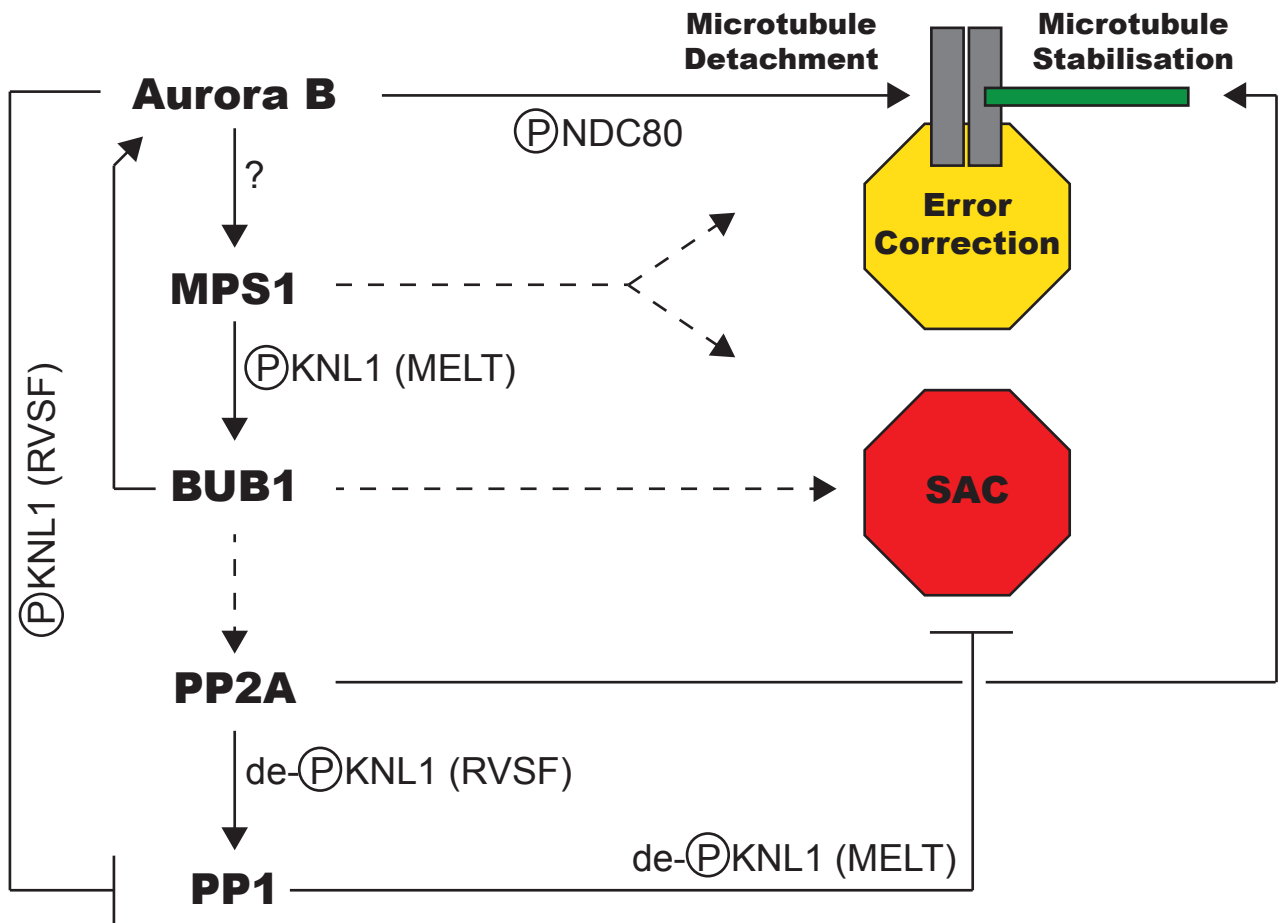


Figure 1: Network model of kinase/phosphatase signaling at kinetochores. A selected set of kinases and phosphatases that control error-correction and the SAC are indicated along with some of their relevant substrates. Solid lines indicate direct connections, while dashed lines indicate indirect ones

Type	Antibody	Species	Dilution	Company	Cat number /reference
Kinase	Aurora B (AIM-1, Clone 6)	Mouse	1:1000	BD Transduction	611083
Kinase	Bub1	Rabbit	1:1000	Bethyl	A300-373A-1
Kinase	Cyclin B1 (Clone GNS1)	Mouse	1:500	Santa Cruz	sc-245
Kinase	Cyclin B1	Rabbit	1:500	Santa Cruz	sc-752
Kinase	MPS1, NT (Clone 3-472-1)	Mouse	1:1000	Millipore	05-682
Kinase	Plk1	Rabbit	1:1000	Custom	(15)
Aurora B autophosphorylation site	p-Aurora B (Thr232)	Rabbit	1:4000	Rockland	600-401-677
Aurora B chromatin substrate	p-Histone H3 (Ser10)	Rabbit	1:10000	Millipore	06-570
Aurora B inner kinetochore substrate	p-CenpA (Ser7)	Rabbit	1:1000	Millipore	07-232
Aurora B outer kinetochore substrate	p-NDC80 (Ser55)	Rabbit	1:1000	Genetex	GTX70017
BUB1 substrate	p-Histone H2A (Thr120)	Rabbit	1:4000	Active motif	39391

CDK1 kinetochore substrate	p-BUBR1 (Ser670)	Rabbit	1:2000	Custom	(12)
MPS1 autophosphorylation site	p-MPS1 (Thr676)	Rabbit	1:1000	Custom	(16)
MPS1 kinetochore substrate	pMELT-KNL1 (pThr943/Thr1155)	Rabbit	1:2000	Custom	(12)
MPS1 kinetochore substrate	pMELT-KNL1 (pThr875)	Rabbit		Custom	(14) (17)
PLK1 substrate	p-BUBR1 (Thr680)	Rabbit	1:1000	Custom	(11)
Unattached kinetochore marker	Mad1 (Clone BB3-8))	Mouse	1:1000	Millipore	MABE867
Kinetochore marker	KNL1	Rabbit	1:1000	Abcam	ab-70537
Kinetochore marker	NDC80 (Clone 9G3)	Mouse	1:5000	Abcam	ab-3613
Kinetochore marker	ACA	Human	1:2000	Fitzgerald	90c-CS1058
Kinetochore marker	CenpC	Guinea pig	1:5000	Caltag Medsystems	PD030

Table 1: A list of kinetochore antibodies that have been verified to work by immunofluorescence

Kinase	Small molecule inhibitor	IC50	[work] in HeLa	ref
Aurora B	ZM447439	130 nM	2 μM	(19)
	Hesperadin	~250 nM	100 nM	(20)
	various others, in (pre)clinical trials			(21)
MPS1	Mps1-IN1	367 nM	10 μM	(22)
	Reversine	98.5 nM	1 μM	(23)
	AZ3146	35 nM	2 μM	(24)
	NMS-P715	65 nM	1 μM	(25)
	Various others, in (pre)clinical trials			
PLK1	BI2536	0.83 nM	100 nM	(26)
	TAL	19 nM	1 μM	(27)
	Various others, in (pre)clinical trials			(21)
BUB1	None			
CDK1	RO-3306	35 nM	10 μM	(28)
Note that various other CDK inhibitors are available, including roscovitine, flavopiridol and olomoucine, but they generally inhibit a broader range of CDK enzymes.				
'shokat' alleles				
AurB-as (L154A;H250Y)	NA-PP1	ND	2 μM	(29)
Mps1-as (M602G and M602A)	23-dMB-PP1/3MB-PP1	ND	1/10 μM	(30, 31)
PLK1-as	3MB-PP1	ND	10 μM	(32)

BUB1: is natural	2OH-BNPP1	250 nM	Only in vitro	(33)
gatekeeper				
mutant (G866)				
CDK1-as	1NM-PP1	ND	10 µM	(34)
(chicken, none				
reported in				
humans)				

Table 2: A list of inhibitors against different kinetochore kinases

Drug	Working concentration (in HeLa cells)	Effect	Uses
Nocodazole	3.3 μ M	Unattached kinetochores	To visualise unattached kinetochores and determine maximal kinase activity (35, 36)
Taxol	1 μ M	Few unattached kinetochores	Generates a few Aurora-B dependent unattached kinetochores due to lack of tension (37)
Nocodazole	20-80 nM	Partial chromosome alignment	To visualise attached kinetochores under tension and unattached or unaligned kinetochores within the same cell.
Noscapine	2-25 μ M	Partial chromosome alignment	To visualise attached kinetochore under tension and unattached or unaligned kinetochores within the same cell.
STLC	10 μ M	Monotelic attachments	To visualise attached and unattached kinetochores within the same cell (using Mad1 to mark unattached kinetochores)
Monastrol	200 μ M	Monotelic attachments or merotelic attachments (after washout)	To visualise attached and unattached kinetochores within the same cell (using Mad1 to mark unattached kinetochores). Monastrol washout leads to increased number of merotelic attachments (i.e. one kinetochore attached to both spindle poles).

MG-132	5 μ M	Complete chromosomal alignment	To visualise attached kinetochores under tension (see note 4).
Pro-TAME	12 μ M	Complete chromosomal alignment	To visualise attached kinetochores under tension without inhibiting proteasome activity (see note 4).

Table 3: A list of Inhibitors that can be used to modulate kinetochore–microtubule attachment status